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Research Report

Cognitive Function After Stopping Folic Acid and DHA Intervention: An Extended Follow-Up Results from the Randomized, Double Blind, Placebo-Controlled Trial in Older Adults with Mild Cognitive Impairment

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Abstract.
Background: Our previously randomized controlled trial (RCT) showed daily oral folic acid (FA), docosahexaenoic acid (DHA) and their combined treatment for 6 months could significantly improve cognitive function in mild cognitive impairment (MCI) individuals.
Objective: This study aimed to evaluate whether this benefit seen in the treatment group would sustain after stopping intervention when patients returned to a real-world.
Methods: RCT (ChiCTR-IOR-16008351) was conducted in Tianjin, China. 160 MCI elders aged ≥60 years were randomly divided into four groups: FA + DHA, FA, DHA, and control. 138 MCI elders who completed the 6-month interventional trial underwent another 6-month follow-up without receiving nutritional therapy. Cognitive performance was measured at 6 and 12 months. Blood amyloid-β peptide (Aβ) and homocysteine (Hcy) related biomarkers were measured at baseline and 6 months.

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Results: In comparison to the end of nutritional therapy, all intervention groups had considerably lower full-scale IQ, arithmetic, and image completion scores during the follow-up period, while the combined intervention and DHA groups had significantly lower picture arrangement scores. Furthermore, after 6-month treatment with FA and FA + DHA, plasma $A\beta_{40}$, $A\beta_{42}$, and Hcy levels were significantly decreased. However, these biomarker levels at the start of follow-up were positively correlated with the degree of cognitive function change during follow-up period.

Conclusions: FA and DHA supplementation enhance cognitive performance in MCI elderly following a six-month intervention by reducing Hcy or Aβ levels. However, their effects on improving cognitive decline are likely to diminish when the intervention is discontinued.

Keywords: Alzheimer's disease, amyloid-β-related biomarkers, docosahexaenoic acid, folic acid, mild cognitive impairment, one carbon metabolism, real world study

INTRODUCTION

Folic acid (FA)[1] and docosahexaenoic acid (DHA),[2] as essential nutrients, play an important role in improving cognitive function of individuals with mild cognitive impairment (MCI). DHA is the main omega-3 polyunsaturated fatty acid (n-3 PUFA), which could promote the development of neurons to maintain brain function and could also exert anti-inflammatory properties to affect the neural membrane plasticity and inflammatory signaling pathways. B vitamins, especially FA, are important cofactors in metabolic pathways that may modulate the homocysteine (Hcy) methylation process to affect cognitive impairment. Epidemiologic studies have observed that low levels of DHA or folate may be associated with increased odds of MCI and Alzheimer's disease (AD).[3,4] However, the prevalence of folate deficiency is above 20% in the Chinese elderly, China has no official FA fortification program and traditional cooking methods tend to cause folate loss from vegetables. Similarly, the consumption of DHA among Chinese elderly was also insufficient, less than 12 mg/day.[5] Meanwhile, our previous study indicated that certain nutrients could improve cognitive abilities of individuals with MCI.[6]

Hcy is an independent risk factors of AD, which causes neuronal apoptosis and increases susceptibility of hippocampal neurons to oxidative damage.[7,8] Folate deficiency significantly reduces SAM/SAH ratios *in vivo* and *in vitro*.[9] PUFAs, particularly DHA, may impact the Hcy level by effecting Methyltetrahydrofolate-reductase (MTHFR) expression. Abnormal amyloid-β (Aβ) metabolism and deposition are pivotal in AD development. Aβ as the hall biomarker of AD leads to excessive activation of microglia, results in neuroinflammation of brain tissue as well as neuronal damage. Reduction in Aβ burden in AD might slow the disease's progression by improving nutrient levels, in particular vitamin B and n-3 PUFA.[10,11] Aβ is obtained from the catabolism of amyloid-β protein precursor (AβPP). We have found that folate reduced Aβ deposition through affecting AβPP metabolism.[12]

In our previous RCT,[6] we discovered that the daily oral administration of FA, DHA, and their combined usage for 6 months significantly enhanced cognitive performance in individuals with MCI. As previously mentioned, none of our participants were taking dietary supplements, suggesting that their regular dietary intake might not have provided adequate amounts of folic acid and DHA. Building upon this, the present study aimed to determine whether the observed cognitive improvements could persist after the intervention was discontinued, as patients returned to the real-world. The primary objective was to evaluate the long-term efficacy and sustainability of the intervention measures. Moreover, we examined the post-intervention changes in one-carbon unit metabolism biomarkers and Aβ biomarkers in participants' blood, which can provide valuable insights into the intervention's impact on these biological markers and its effects on mild cognitive impairments in older adults. Ultimately, this research aimed to contribute scientific evidence supporting intervention strategies and holds clinical significance for the long-term management and prevention of cognitive impairments in older individuals.

MATERIALS AND METHODS

Study design, participants, and follow-up

In the preliminary stages, we have conducted a two-center randomized controlled trial (RCT) and discovered that daily taking FA, DHA, and their combination for 6 months significantly improved cognitive performance of individuals with MCI.

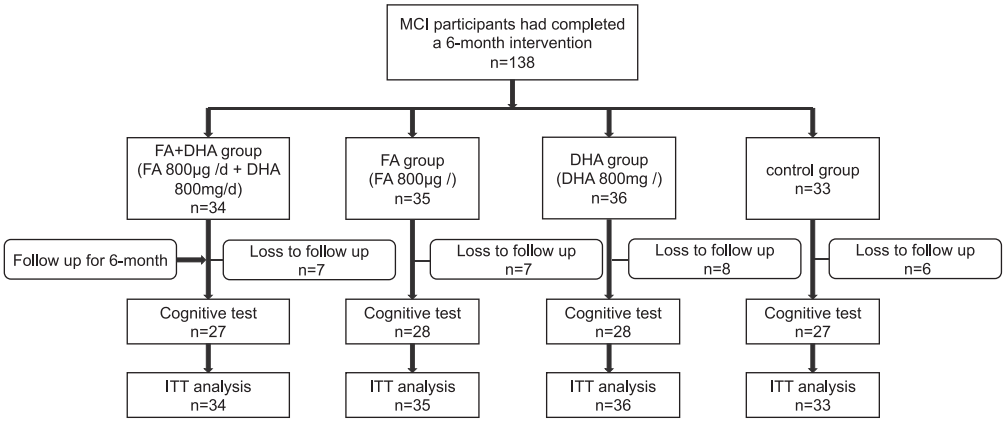

Fig. 1. Flow diagram of enrolment and follow-up in the study.

Based on this conclusion, one of these communities was selected for an extended follow-up study to ascertain whether the intervention's effect can be maintained once patients return to a real-world situation after the intervention ends. Each patient who successfully completed the final intervention for the RCT was eligible to start the extended follow-up period. The prolonged follow-up period started at the end of the intervention when all trial-related procedures were stopped, and patients were followed up for an additional 6 months in a real-world setting. At the end of the follow-up period, cognitive functions of the MCIs were evaluated.

The study population was recruited from the Hujiayuan Community Health Service Center in the Binhai New Area of Tianjin, China between March and June 2016. The following inclusion criteria were applied: 1) over the age of 60; 2) free of mental disorders (such as major depression, schizophrenia, bipolar disorders, etc.); 3) not using any medications known to affect nutrition status, folate metabolism (including anti-platelet medications), fatty acid composition metabolism, or cognitive function in the three months preceding recruitment; and 4) in generally good health, ambulatory, and with adequate hearing and vision for compliance with testing procedures. The randomization sequence and intervention procedures were previously described in detail. In short, 160 MCI patients were enrolled in this study and randomly allocated to the FA+DHA group, FA group, DHA group, or placebo group ($n=40$ per group). 138 MCI patients (FA + DHA group: $n=34$, FA group: $n=35$, DHA group: $n=36$, control group: $n=33$) completed the treatment strategy and were included in the extended follow-up period, with 110 (FA + DHA group: $n=27$, FA group: $n=28$, DHA group: $n=28$, control group: $n=27$) ultimately followed up. The flow of study participants is shown in Fig. 1.

All participants have provided written informed consent. This study adheres to the principles of the Declaration of Helsinki. The trial number was registered as ChiCTR-IOR-16008351 (https://www.chictr.org.cn/showproj.html?proj=12439), the protocol for main RCT and follow-up study were approved by the Ethics Committee of Tianjin Medical University.

Data collection during follow-up

Participants who met the criteria and had successfully completed the FA and DHA intervention for 6 months were recruited in this follow-up study. The primary outcome of the extended follow-up period was cognitive performance, consistent with the RCT.[6] We assessed cognitive function using various domains from the Chinese version of the Wechsler Adult Intelligence Scale-Revised (WAIS-RC) at the beginning and end of the follow-up period. Throughout this period, communication with the participants was maintained via telephone, face-to-face interviews, or email, enabling us to monitor and document any adverse events or unexpected occurrences during the follow-up process. Additionally, participants' fasting venous blood samples were collected at the start of follow-up, which were used for the measurement of peripheral one-carbon unit related metabolite levels, β-amyloid protein, and its associated metabolic indicators.

Cognitive function assessment

The cognitive function was assessed by trained physicians using the short form IQ and index scores derived from the WAIS-RC, as previously described in our published work.[6] This assessment included a selection of six verbal subtests and five performance subtests, with specific subtests chosen to reduce testing time and promote patient engagement. Age-appropriate norms from Chinese standardization were applied to calculate the IQ and index scores.

Blood collection and analysis

Fasting venous anti-coagulant blood samples were collected in the morning at baseline and 6 months of intervention, and then centrifuged at 3,000 rpm and 1,700 g for 10 min and then stored at –80°C until analysis. EDTA anticoagulant was used to separate plasma samples, and separating gel coagulant was used to collect serum samples. Serum Hcy, plasma SAM, and SAH levels were measured using a DIRUI CS-T300 automatic biochemistry analyzer (China), using the enzymatic conversion method. Kits were purchased from Ningbo Medical System Biotechnologies, Inc. (China). Protein levels of $A\beta_{40}$, $A\beta_{42}$, and AβPP were assessed using an enzyme-linked immunosorbent assay kit (Wuhan Huamei Bioengineering Co., Ltd., China). Gene expression levels of AβPP mRNA, β-secretase1-mRNA (BACE1-mRNA), Presenilin1-mRNA (PS1-mRNA), and Presenilin2-mRNA (PS2-mRNA) was quantified by real-time PCR. The assay was performed using the Roche LightCycler 480 sequence detector (Roche, Mannheim, Germany).

Statistical analysis

Data were expressed as the mean ± standard deviation. The baseline characteristics of the intervention and placebo groups were compared by one-way analysis of variance for continuous variables, with post hoc comparison using the Bonferroni test for multiple comparisons. Intention-to-treat (ITT) analyses were performed using the expectation–maximization algorithm (EM) for subjects who were lost to follow-up with missing data. The ITT participants consisted of all randomized patients who completed the intervention. Generalize estimating equation (GEE) with an exchangeable working correlation matrix were used to analyze changes in cognitive function at 0–6 months and 6–12 months, and blood biochemical indicators at 0–6 months. The results are presented as estimates (with 95% confidence intervals [CIs]) of the difference between the intervention and control groups for time periods combined, for each test of cognition. To facilitate easier comparison, the differences between the intervention groups and the placebo group for each variable were estimated, with adjustments for baseline values, lifestyle, and medical history in the first model, and further adjustments for sex and education in the second model. All statistical tests were two-sided, and a p-value of <0.05 was considered to indicate a significant statistical difference. SPSS (IBM Corp.) was used to analyze the data.

Pearson correlations were utilized to examine the relationship between cognitive performance and blood biochemical indicators at the start of the follow-up. Additionally, the correlation between the extent of cognitive performance change (D-values=WAIS-RC scores at 12 month – WAIS-RC scores at 6 month) and biochemical indicators at the start of follow-up was also analyzed by using Pearson correlations. Heat maps of correlations were generated and analyzed by using R Studio.

RESULTS

Characteristics of participants

160 participants were randomly equally assigned to four groups. Following a 6-month intervention, 138 MCIs (FA + DHA group: $n = 34$, FA group: $n = 35$, DHA group: $n = 36$, control group: $n = 33$) completed the intervention and were recruited into the extended follow-up period. Table 1 lists the characteristics in four groups. There was no significant difference in the demographic traits, medical history and lifestyle status ($p > 0.05$).

Changes in cognitive performance

As shown in Table 2, daily oral FA and/or DHA supplementation for 6 months improve cognitive performance in MCI individuals. There were significant improvements in the scores of full-scale IQ, arithmetic and digit span in 3 intervention groups ($p < 0.01$), compared with the placebo group. DHA supplementation for 6 months improved the information, block design and picture arrangement scores ($p < 0.01$). The picture completion scores were considerably improved by FA alone or in combination with DHA supplementation ($p < 0.05$).

Table 1
Baseline characteristics of the study population

Profile	FA + DHA group ($n = 34$)	FA group ($n = 35$)	DHA group ($n = 36$)	Control group ($n = 33$)	p
Demography					
Age at screening (y)	66.74 ± 5.79	67.51 ± 5.07	70.17 ± 6.54	68.30 ± 6.38	0.098
Sex, female, n (%)	20(58.8)	23(65.7)	25(69.4)	19(57.6)	0.700
Total education (y)	6.88 ± 3.09	7.11 ± 2.53	6.08 ± 3.08	6.73 ± 3.00	0.492
Lifestyle					
Smoker – ever	7(20.6)	8(22.9)	6(16.7)	6(18.2)	0.316
Alcohol – ever	11(32.4)	11(31.4)	5(13.9)	7(21.2)	0.556
BMI (kg/m^2)	24.92 ± 2.27	26.49 ± 3.43	24.98 ± 2.58	24.31 ± 3.17	0.017
Medical history					
Diabetes	5(14.7)	11(31.4)	10(27.8)	4(12.1)	0.139
TIA/stroke	0	6(17.1)	3(8.3)	2(6.1)	0.068
Cardiopathy	7(20.6)	4(11.4)	7(19.4)	8(24.2)	0.580
Hypertension	25(73.5)	21(60.0)	23(63.9)	17(51.5)	0.311

FA, folic acid; DHA, docosahexaenoic acid.

However, after 6 months of cessation of the intervention, most of the above indicators decreased. The full-scale IQ, arithmetic and picture completion scores in all 3 intervention groups were considerably lower than the scores at 6 months ($p < 0.05$). In both the combination intervention group and the DHA group, picture arrangement scores at 12 months were considerably lower than those at 6 months ($p < 0.01$). Additionally, there was no discernible decline in digit span.

According to the GEE analysis, we found that the FA/DHA intervention groups did not experience a significant sustained improvement effect on the neuropsychological tests following the cessation of intervention for six months.

Correlations of cognitive performance and blood biomarkers

Figure 2 demonstrates the correlations between cognitive performance and blood biomarkers. Figure 2A presents result of a correlation analysis cognitive performance and methylation related biomarkers related biomarkers at 6 months, and Fig. 2B reflects the correlation between cognitive performance and Aβ-related biomarkers at 6 months. Figure 2C and 2D respectively show a relationship between the extent of cognitive performance change during follow-up and methylation/Aβ-associated biomarkers at the start of follow-up.

The findings revealed that, after 6 months FA and/or DHA supplementation, arithmetic score was adversely connected with Hcy levels, and picture completion score was negatively correlated with SAH levels ($p < 0.01$). FIQ, arithmetic and picture completion scores were found to be negatively associated to $A\beta_{42}$ and $A\beta_{40}$ levels ($p < 0.05$). Picture completion score was adversely correlated to SAH and AβPP levels but positively related to PS1-mRNA expression ($p < 0.05$).

After 6-month follow-up without FA/DHA supplement, participants exhibited an overall decrease in cognitive performance (Table 2). The D-values for cognitive scores over the 6-month interval were calculated, and the correlation between these D-values and biomarker levels at 6 months was investigated. We found a general positive correlation as shown in Fig. 2C and 2D, indicating that lower initial biomarker levels were associated with greater declines in cognitive scores.

Changes in methylation related biomarkers

Serum Hcy levels and plasma SAM and SAH were collected and tested in 138 individuals with MCI at baseline and at 6 months, and the SAM/SAH ratio was calculated (Table 3). After intervention, serum Hcy levels in the FA+DHA group and FA group were significantly lower than that in control group ($p < 0.05$). Further, the SAM levels in the FA+DHA group and FA group were significantly higher than that in control group ($p < 0.05$). In addition, the SAM/SAH ratio was markedly elevated in the FA group compared with control group ($p < 0.05$).

Changes in Aβ related biomarkers

The GEE analysis revealed that over the 6-month intervention, FA supplementation alone, and in combination with DHA led to a significant reduction in $A\beta_{42}$, $A\beta_{40}$, and AβPP levels ($p < 0.05$), while after 6-month supplementation the $A\beta_{40}$ levels in

Table 2
The changes of WAIS-RC tests results

Test of cognition	Groups	*n*	Time*			β (95%CI)†	*p*	β (95%CI)‡	*p*
			0 month	6 months	12 months				
Full-scale IQ	FA + DHA	34	101.26 ± 3.03	102.37 ± 2.07	101.04 ± 2.30	–2.328 (–4.131, –0.525)	0.011	2.273 (0.635, 3.911)	0.007
	FA	35	102.34 ± 3.51	104.54 ± 2.50	100.37 ± 1.54	–5.164 (–6.954, –3.374)	<0.001	3.349 (1.722, 4.975)	<0.001
	DHA	36	100.47 ± 3.89	102.24 ± 2.64	100.10 ± 3.78	–3.136 (–4.914, –1.358)	0.001	2.926 (1.311, 4.542)	<0.001
	Control	33	101.98 ± 2.87	100.82 ± 3.32	101.82 ± 1.70	0 (ref)		0 (ref)	
Information	FA + DHA	34	8.26 ± 1.11	9.15 ± 0.66	8.62 ± 1.05	–0.439 (–1.005, 0.128)	0.129	0.428 (–0.173,1.028)	0.163
	FA	35	8.20 ± 1.32	9.23 ± 1.11	8.63 ± 0.81	–0.509 (–1.072,0.054)	0.076	0.574 (–0.022,1.170)	0.059
	DHA	36	7.64 ± 1.33	8.97 ± 0.77	8.33 ± 0.68	–0.548 (–1.107,0.011)	0.055	0.879 (0.287,1.471)	0.004
	Control	33	8.39 ± 1.00	8.85 ± 0.83	8.76 ± 0.61	0 (ref)		0 (ref)	
Arithmetic	FA + DHA	34	7.94 ± 0.42	9.82 ± 0.63	8.35 ± 0.49	–1.501 (–2.016, –0.986)	<0.001	1.731 (1.211,2.250)	<0.001
	FA	35	8.23 ± 1.06	9.29 ± 0.83	8.60 ± 0.65	–0.716 (–1.227, –0.205)	0.006	0.906 (0.390,1.422)	0.001
	DHA	36	7.64 ± 1.10	9.19 ± 0.71	8.36 ± 0.59	–0.864 (–1.371, –0.356)	0.001	1.404 (0.892,1.916)	<0.001
	Control	33	8.18 ± 0.92	8.33 ± 1.24	8.36 ± 0.60	0 (ref)		0 (ref)	
Digit span	FA + DHA	34	8.62 ± 1.35	9.26 ± 1.08	8.68 ± 0.84	–0.103 (–0.830,0.623)	0.780	1.253 (0.733,1.773)	<0.001
	FA	35	9.17 ± 1.72	9.17 ± 1.72	8.31 ± 0.68	–0.372 (–1.094,0.349)	0.312	0.606 (0.090,1.123)	0.021
	DHA	36	8.36 ± 1.46	9.11 ± 1.30	8.94 ± 1.09	0.318 (–0.398,1.035)	0.384	1.356 (0.843,1.869)	<0.001
	Control	33	9.79 ± 1.80	9.18 ± 1.19	8.70 ± 0.95	0 (ref)		0 (ref)	
Picture completion	FA + DHA	34	15.18 ± 0.83	14.24 ± 0.74	14.26 ± 0.51	–1.304 (–2.131, –0.477)	0.002	0.756 (0.064,1.447)	0.032
	FA	35	15.69 ± 0.90	15.69 ± 0.90	14.00 ± 0.59	–3.019 (–3.841, –2.198)	<0.001	1.697 (1.010,2.384)	<0.001
	DHA	36	15.81 ± 0.86	14.19 ± 0.71	13.89 ± 2.45	–1.639 (–2.455, –0.823)	<0.001	0.086 (–0.596,0.768)	0.805
	Control	33	15.09 ± 1.07	13.39 ± 1.50	14.73 ± 0.57	0 (ref)		0 (ref)	
Block design	FA + DHA	34	7.18 ± 0.72	8.03 ± 0.52	7.82 ± 0.39	0.127 (–0.244,0.499)	0.501	0.429 (–0.066,0.924)	0.090
	FA	35	7.80 ± 1.13	8.06 ± 0.68	7.71 ± 0.52	–0.010 (–0.378, 0.359)	0.960	–0.167 (–0.659,0.324)	0.505
	DHA	36	6.97 ± 1.42	8.17 ± 0.66	7.78 ± 0.49	–0.056 (–0.422, 0.310)	0.766	0.770 (0.282,1.258)	0.002
	Control	33	7.73 ± 0.76	8.15 ± 0.57	7.82 ± 0.47	0 (ref)		0 (ref)	
Picture arrangement	FA + DHA	34	12.35 ± 1.01	13.26 ± 0.75	12.15 ± 0.78	–0.542 (–0.964, –0.120)	0.012	0.215 (–0.180,0.610)	0.287
	FA	35	12.31 ± 0.97	12.71 ± 1.20	12.06 ± 0.80	–0.081 (–0.501, 0.338)	0.704	–0.297 (–0.689,0.095)	0.138
	DHA	36	12.19 ± 0.95	13.31 ± 0.89	12.17 ± 0.74	–0.563 (–0.980, –0.147)	0.008	0.414 (0.024,0.804)	0.037
	Control	33	12.03 ± 0.85	12.73 ± 1.01	12.15 ± 0.88	0 (ref)		0 (ref)	

*Plus-minus value are means ± SD. In all tests, higher scores indicate better function. Data at baseline reflect test results from participants in each group. †The difference of 12 months comparing with 6 months adjusted for baseline value and age, education level and gender. ‡The difference of 6 months comparing with 0 month adjusted for baseline value and age, education level and gender. FA, folic acid; DHA, docosahexaenoic acid.

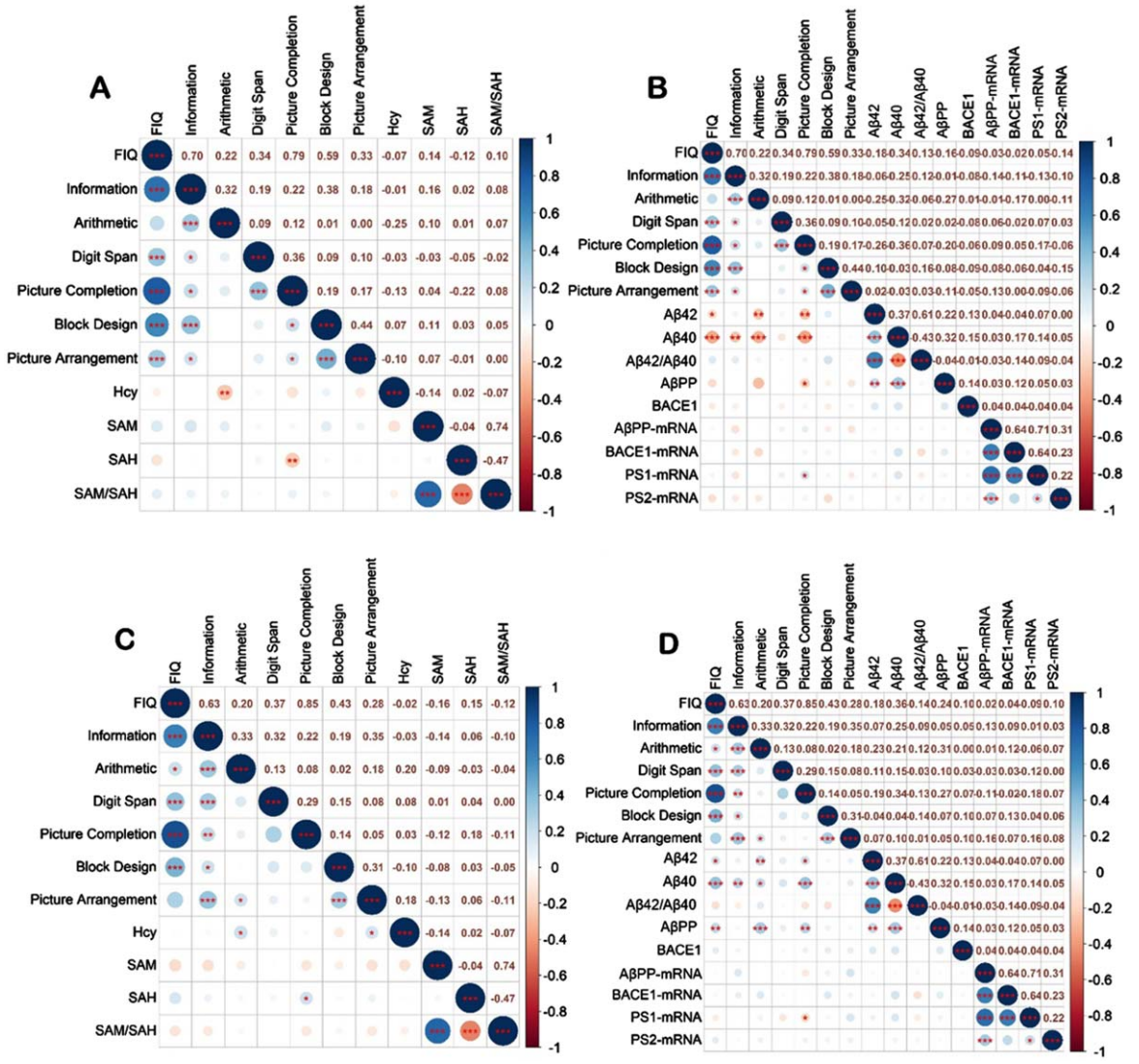

Fig. 2. Correlation heat map. A) Correlation heat map of cognitive functions and methylation related biomarkers at 6 months. B) Correlation heat map of cognitive functions and Aβ-related biomarkers 6 months. C) Correlation heat map of D-values of cognitive functions during follow-up and methylation related biomarkers at 6 months. D) Correlation heat map of D-values of cognitive functions during follow up and Aβ-related biomarkers at 6 months. D values = WAIS-RC scores at 12 month – WAIS-RC scores at 6 months. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

the DHA group were significantly lower than that in the control group ($p<0.05$). However, no significant difference was observed in the $A\beta_{40}/A\beta_{42}$ ratio, BACE1 levels and the expression of AβPP-mRNA, BACE1-mRNA, PS1-mRNA and PS2-mRNA among the groups (Table 4).

DISCUSSION

Various research, including our previous study, have documented the crucial roles that FA and DHA play in maintaining overall health and enhancing cognitive performance.[2,3,6] In this two-stage real-world follow-up study, during the RCT phase, our results demonstrated that 6 months of supplementation with FA combined with DHA improves cognitive performance in older adults with MCI. After the cessation of the intervention, participants underwent a real-world setting for 6 months devoid of any intervention. Our results indicate that the cognitive benefits attributed to FA and DHA during the primary RCT period exhibit a discernible decline trend in cognitive performance

Table 3
The change of levels of methylation related biomarkers

Item	Groups	*n*	Time*		Fully adjusted difference (95%CI) †	*p*
			0 month	6 months		
Hcy	FA + DHA	34	16.58 ± 5.34	12.48 ± 7.77	–6.512 (–10.574, –2.450)	0.002
(μmol/ml)	FA	35	16.84 ± 4.99	15.10 ± 5.09	–4.155 (–8.188, –0.122)	0.043
	DHA	36	16.96 ± 4.06	18.07 ± 8.76	–1.310 (–5.316, 2.696)	0.522
	Control	33	17.70 ± 6.66	20.12 ± 4.54	0 (ref)	
SAM	FA + DHA	34	67.70 ± 27.51	81.92 ± 34.03	23.93 (1.86, 46.00)	0.034
(μg/L)	FA	35	68.75 ± 29.24	92.98 ± 53.84	33.94 (12.03, 55.86)	0.002
	DHA	36	72.50 ± 24.95	80.24 ±32.80	17.45 (–4.32, 39.22)	0.116
	Control	33	76.95 ± 21.31	67.24 ± 21.32	0 (ref)	
SAH	FA + DHA	34	20.18 ± 8.66	20.16 ± 6.53	–0.16 (–5.67, 5.36)	0.956
(μg/L)	FA	35	21.48 ± 9.75	18.62 ± 8.97	–3.00 (–8.47, 2.48)	0.283
	DHA	36	24.16 ± 6.19	21.85 ± 6.39	–2.45 (–7.89, 2.99)	0.378
	Control	33	21.27 ± 10.15	21.41 ± 9.36	0 (ref)	
SAM/SAH	FA + DHA	34	3.52 ± 1.69	4.25 ± 2.06	0.97 (–1.18, 3.12)	0.378
(μg/L)	FA	35	3.46 ± 2.05	6.20 ± 7.25	2.98 (0.83, 5.13)	0.007
	DHA	36	3.14 ± 1.31	4.06 ± 2.28	1.15 (–0.95, 3.24)	0.283
	Control	33	3.75 ± 1.62	3.56 ± 1.93	0 (ref)	

*Plus-minus value are means ± SD. Data at baseline reflect test results from participants in each group. †The difference is adjusted for baseline value and age, education level and gender. FA, folic acid; DHA, docosahexaenoic acid.

after 6 months of intervention cessation. This shows that the effects of FA and DHA treatments on preserving long-term cognitive performance may be limited.

The decline in cognitive performance after the withdrawal of folic acid and DHA intervention can be attributed to multiple factors. One possible important factor may have been the decrease in effective concentrations in the brain after withdrawal.[13,14] Participants in this trial received only folic acid and DHA therapies; no dietary or lifestyle recommendations were made. FA is a water-soluble vitamin which has a restricted amount of storage within the body. A study involving daily supplementation of 1 mg of FA over a continuous period of 2 years indicated that it takes approximately 1.5–2 years for FA concentrations in the blood to reach a stable state, depending on the dosage of FA.[15] However, there is no official folic acid fortification program in China, and conventional cooking techniques frequently cause vegetables to lose their folic acid content. DHA is a crucial long-chain polyunsaturated fatty acid, which can be stored within the body and is widely distributed throughout brain tissue.[16,17] The amount of DHA consumed affects how much of it is depleted in the plasma, which in the case of DHA deficiency, ultimately results in a considerable drop in DHA levels in the brains of both rats and humans.[18,19] Participants in this trial only received an intervention consisting of folic acid and DHA; no dietary or lifestyle recommendations were made. In the previous study[6] and the present study, there were no significant changes in plasma FA and DHA levels in the placebo group, and it can be assumed that participants' dietary intake levels of the nutrients were relatively stable during the study period. Since the participants followed their initial dietary and lifestyle practices throughout the experiment, it is possible that the DHA and folate levels might return to baseline when the intervention was terminated.

To further explore the possible mechanism of cognitive performance decline after cessation of intervention, we detected biomarkers related to one-carbon and Aβ metabolism. As previously reported, the level of Hcy and Aβ related biomarkers is associated with cognitive performance in patients with AD or MCI.[20] Hcy can interfere with the metabolism and degradation of Aβ, leading to Aβ accumulation.[21] Blood FA status has been identified as a crucial modulating factor for Hcy levels.[22] Previous epidemiological studies have shown that FA can reduce plasma levels of one-carbon biomarkers, thereby exerting neuroprotective effects against AD or MCI.[23,24] PUFAs have been found to modulate the gene expression of the critical enzymes for Hcy metabolism.[25] DHA has been shown to potentially alleviate disorders such as AD in older people by reducing Hcy levels and subsequently downregulating neuroamide levels.[26,27] Meanwhile, FA deficiency has been demonstrated to increase the accumulation of Aβ peptides in the brains of APP/PS1 mice.[28,29] DHA has also been proven to inhibit the production and aggregation of Aβ peptides.[30–32] Additionally, both FA and DHA can protect hippocampal neurons from the toxic damage

Table 4
The change of levels of Aβ-related biomarkers

Item	Groups	*n*	Time*		Fully adjusted difference (95%CI)†	*p*
			0 month	6 months		
$A\beta_{42}$ (pg/ml)	FA + DHA	34	217.61 ± 92.03	180.30 ± 68.10	–98.06 (–154.43, –41.69)	0.001
	FA	35	206.26 ± 93.79	170.64 ± 65.85	–96.37 (–152.34, –40.40)	0.001
	DHA	36	198.89 ± 103.36	257.17 ± 72.71	–2.47 (–58.06, 53.13)	0.931
	Control	33	207.44 ± 104.17	268.19 ± 65.57	0 (ref)	
$A\beta_{40}$ (pg/ml)	FA + DHA	34	147.24 ± 39.85	101.66 ± 25.44	–86.32 (–126.09, –46.55)	<0.001
	FA	35	120.02 ± 129.87	86.93 ± 18.30	–73.84 (–113.33, –34.35)	<0.001
	DHA	36	117.77 ± 33.75	117.95 ± 30.61	–40.57 (–79.79, –1.35)	0.043
	Control	33	126.44 ± 40.49	167.19 ± 67.70	0 (ref)	
$A\beta_{42}$/ Aβ40	FA + DHA	34	1.48 ± 0.52	1.82 ± 0.72	0.29 (–0.40, 0.98)	0.407
	FA	35	2.30 ± 1.73	2.04 ± 0.85	–0.31 (–0.99, 0.37)	0.368
	DHA	36	1.76 ± 0.94	2.25 ± 0.67	0.45 (–0.23, 1.13)	0.193
	Control	33	1.84 ± 1.28	1.89 ± 0.93	0 (ref)	
AβPP (ng/ml)	FA + DHA	34	1850.92 ± 635.00	1382.59 ± 954.71	–918.59 (–1483.87, –353.30)	0.001
	FA	35	1905.64 ± 877.17	1321.20 ± 1003.06	–1034.70 (–1595.99, –473.41)	<0.001
	DHA	36	2031.41 ± 676.61	1941.35 ± 764.94	–540.31 (–1097.81, 17.18)	0.057
	Control	33	1854.39 ± 1072.34	2304.65 ± 854.79	0 (ref)	
BACE1 (ng/ml)	FA + DHA	34	41.71 ± 41.76	37.59 ± 23.86	–5.37 (–25.40, 14.66)	0.599
	FA	35	42.27 ± 35.13	37.62 ± 25.46	–5.91 (–26.08, 14.26)	0.566
	DHA	36	47.68 ± 25.69	48.74 ± 28.29	–0.19 (–20.51, 20.13)	0.985
	Control	33	40.79 ± 22.76	42.05 ± 27.07	0 (ref)	
AβPP-mRNA	FA + DHA	34	55.27 ± 32.93	28.24 ± 15.23	–12.40 (–28.36, 3.56)	0.128
	FA	35	53.38 ± 16.06	35.54 ± 25.82	–3.39 (–19.49, 12.71)	0.680
	DHA	36	53.65 ± 27.79	32.11 ± 19.67	–6.81 (–22.84, 9.22)	0.405
	Control	33	53.62 ± 14.33	38.83 ± 23.50	0 (ref)	
BACE1-mRNA	FA + DHA	34	3.64 ± 3.21	1.59 ± 0.95	–1.28 (–2.66, 0.11)	0.070
	FA	35	3.15 ± 1.28	1.91 ± 1.34	–0.45 (–1.84, 0.95)	0.529
	DHA	36	3.50 ± 3.47	1.60 ± 0.91	–1.11 (–2.50, 0.28)	0.117
	Control	33	3.30 ± 1.21	2.52 ± 1.57	0 (ref)	
PS1-mRNA	FA + DHA	34	39.58 ± 21.47	33.89 ± 17.99	–12.12 (–28.81, 4.57)	0.155
	FA	35	39.30 ± 13.89	45.50 ± 37.04	–0.45 (–17.35, 16.44)	0.958
	DHA	36	38.68 ± 19.01	37.24 ± 27.58	–7.94 (–24.71, 8.83)	0.353
	Control	33	40.69 ± 22.63	47.12 ± 29.93	0 (ref)	
PS2-mRNA	FA + DHA	34	4.30 ± 7.33	1.42 ± 2.38	–0.96 (–5.26, 3.35)	0.664
	FA	35	4.27 ± 12.88	2.11 ± 2.85	–0.22 (–4.55, 4.12)	0.923
	DHA	36	3.15 ± 3.03	1.93 ± 3.48	0.85 (–3.47, 5.17)	0.699
	Control	33	4.83 ± 6.04	2.90 ± 3.86	0 (ref)	

*Plus-minus value are means ± SD. Data at baseline reflect test results from participants in each group. †The difference is adjusted for baseline value and age, education level and gender. FA, folic acid; DHA, docosahexaenoic acid.

caused by Aβ, through reducing oxidative stress and inflammation induced by Aβ.[31,33] These results suggest that combined supplementation of FA and DHA may affect the one-carbon metabolic pathway, which in turn affects Aβ synthesis and accumulation. In this investigation, we discovered a negative correlation between cognitive performance and the levels of Hcy, SAH, and Aβ after six months of intervention. While, the lower the levels of Hcy and Aβ before follow-up (after 6 months FA or DHA treatment), the faster the rate of cognitive deterioration after terminating nutritional intervention. The potential cause was that the levels of Hcy and Aβ were unable to maintain at a lower level. Following the removal of folic acid and DHA, the regulatory effects may diminish or vanish, and levels of Hcy and Aβ may progressively revert to pre-intervention levels.

It is worth noting that compared to the baseline data after 6 months of intervention, the reduction in Hcy and Aβ related biomarkers was mainly related to folic acid intervention rather than DHA intervention alone. At the current intervention dose and time, DHA intervention alone, although improving cognitive performance of MCI elderly, did not significantly decrease Hcy or Aβ levels. While folic acid and DHA had different effects on Hcy, these variations were consistent with findings from other studies.[34] Supplementing with higher amount of DHA (2 g/day for 24 months) can reduce Aβ levels in MCI patients.[2] Moreover, short-term DHA

supplementation improving cognitive performance primarily by reducing inflammatory factor levels.[6] In the present study, folic acid and combination intervention significantly increased plasma SAM levels and decreased AβPP and Aβ levels, but there was no significant difference in the expression of Aβ-related mRNAs. Aβ is predominantly synthesized in the brain and effluxes to the periphery,[35] and Aβ peptide metabolism in the central-brain and peripheral is strongly interconnected.[36] In our previous studies, folic acid status has been found to modulate Aβ production by affecting PS1 and BACE1 mRNA expression in the brain.[12,28] However, there was no correlation between the plasma levels of BACE1 (mRNA and protein) and cognitive performance in AD and health elder in other studies.[37] Therefore, we hypothesized that plasma Aβ-associated mRNAs may not accurately represent Aβ synthesis in the brain, and plasma Aβ-associated mRNAs may not corelated to cognitive performance.

Our study has several limitations. Firstly, a small number of individuals being lost to follow-up at the start of the follow-up period may have resulted in insufficient power to detect significant differences. Secondly, the polymorphism of *APOE* and *MTHFR* genes may be related to the decline in cognitive performance and folate metabolism, but they were not detected in this study. Thirdly, blood samples were not collected from participants and relevant indicators were not detected at the end of the follow-up period. Finally, participants who undergo a certain intervention may have adopted behaviors to compensate or offset the effect of the intervention, but this study did not evaluate such compensatory behaviors that could pose a risk.

In conclusion, our findings indicate that although elderly patients with MCI showed enhancements in cognitive performance after a consistent 6-month administration of oral FA and/or DHA, discontinuation of the intervention may result in a subsequent decline in cognitive performance compared to the end of the intervention period. Therefore, sustained administration of FA and DHA supplements or dietary modifications are necessary to maintain the ameliorative effects on cognitive deterioration.

AUTHOR CONTRIBUTIONS

Dong Bai (Data curation; Investigation); Junting Fan (Formal analysis; Investigation; Writing – original draft); Mengyue Li (Data curation; Investigation; Writing – original draft); Cuixia Dong (Formal analysis); Yiming Gao (Investigation); Min Fu (Formal analysis); Qianfeng Liu (Writing – original draft); Huan Liu (Funding acquisition; Project administration; Supervision; Writing – review & editing).

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

DATA AVAILABILITY

The data supporting the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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